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Chronic lymphocytic leukaemia: the role of T cells in a B cell disease

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Short title: Role of T cells in CLL

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Summary

Chronic lymphocytic leukaemia (CLL) has long been thought to be an immunosuppressive disease and abnormalities in T cell subset distribution and function have been observed in many studies. However, the role of T cells (if any) in disease progression remains unclear and has not been directly studied. This has changed with the advent of new therapies such as CAR-T cells which actively use retargeted patient derived T cells as "living drugs" for CLL. However complete responses are relatively low (~26%) and recent studies have suggested the differentiation status of patient T cells before therapy may influence efficacy. Non-chemotherapeutic drugs such as Idelalisib and Ibrutinib also have an impact on T cell populations in CLL patients. This review will highlight what is known about T cells in CLL during disease progression and after treatment and discuss the prospects of using T cells as predictive biomarkers for immune status and response to therapy.

Keywords# T cells, immunotherapy, leukaemia, CLL

Introduction

The focus for research in CLL has always been to understand the mechanisms that contribute to the expansion and persistence of malignant B cells. Under normal circumstances, B cells play a vital role in adaptive immunity and the transformation of these cells in CLL has a major impact upon susceptibility to infection and response to vaccines (Sinisalo *et al*, 2001). But it is often overlooked that CLL also has profound effects on the other arm of the adaptive immune system, T cells. The massive expansion of malignant B cells is accompanied by expansions of T cells (Catovsky *et al*, 1974; Kay *et al*, 1979; Catovsky *et al*, 1981); counts for both CD4 and CD8 T cells are increased in CLL patients (Mills & Cawley, 1982; Herrmann *et al*, 1982) relative to healthy controls.

These observations were made several decades ago but despite many follow-up studies, the causes of the T cell expansion remain unknown. The link between T cell expansion and progressive disease has led to suggestions that the T cells are tumour specific (Totterman *et al*, 1989). However, despite many putative tumour antigens being proposed (Ramsay & Gribben, 2008; Nunes *et al*, 2011; Cobbold *et al*, 2013), there are no strong antigens that are shared between all CLL patients, yet also absent from normal tissue (Ramsay & Gribben, 2008). Relative to other cancers, CLL has a low mutation rate (Alexandrov *et al*, 2013), so it is unlikely that strong T cell responses are being generated against mutational neo-antigens, as reported for melanoma (Ott *et al*, 2017).

B cells are considered as professional antigen presenting cells, but CLL cells have poor antigen presentation function. This appears to be due to an inability to form activating immune synapses with T cells (Ramsay *et al*, 2008) and this, together with the presence of immunosuppressive molecules (Pallasch *et al*, 2009; Kretz-Rommel *et al*, 2007), results in impaired function of T cells (Görgün *et al*, 2005). Patient T cells do have the capacity to be activated *ex vivo* and defects in immune synapses can be overcome by treatment with lenalidomide (Ramsay *et al*, 2008) or use of bi-specific anti-CD3xCD19 antibodies (Wong *et al*, 2013). Furthermore T cells derived from patients can support the growth of CLL cells *in vitro* (Os *et al*, 2013; Catakovic *et al*, 2017) and *in vivo* when co-transferred into immunodeficient mice (Bagnara *et al*, 2011). However, it remains unclear whether the T cell expansion observed during disease is a reactive process driven by tumour cells and whether these T cells can promote disease.

Since the original observations about T cells in CLL, there has been an explosion of knowledge about T cells. This has been driven by an increasing appreciation of the role they play in maintaining health and by advances in technology that allow analysis at the single

cell level. For example multi-parameter flow cytometry has allowed definition of discrete T cell subsets with different functions and stages of differentiation (Appay *et al*, 2008) (Fig 1). There is also an expanding universe of T cells that are classified as "unconventional", such as $\gamma\delta$ T cells, that have been little studied in CLL (Coscia *et al*, 2012).

This review will bring together current concepts in T cell immunology and many clinical studies of conventional CD4 and CD8 T cells in CLL (summarised in Table 1 and Table 2) to discuss whether additional knowledge about T cells (phenotype, function, differentiation status) can be useful to Haematologists.

T cell abnormalities in CLL

Expansion of CD8 T cells and inverted CD4:CD8 ratios

T cell abnormalities in CLL were first documented over 40 years ago (Catovsky *et al*, 1974; Kay *et al*, 1979) but follow up studies gained more precision with the advent of monoclonal antibodies against CD4 and CD8 (Matutes *et al*, 1981; Platsoucas *et al*, 1982; Herrmann *et al*, 1982; Mills & Cawley, 1982). These early studies described increased proportions of CD8 T cells relative to CD4 T cells, resulting in an inversion of the normal CD4:CD8 ratio (Platsoucas *et al*, 1982; Herrmann *et al*, 1982; Mills & Cawley, 1982). There appears to be an expansion of the entire T cell compartment in CLL (Catovsky *et al*, 1974; Platsoucas *et al*, 1982; Roth *et al*, 2008), but with preferential expansion of CD8 T cells relative to CD4 T cells (Platsoucas *et al*, 1982; Herrmann *et al*, 1982; Mills & Cawley, 1982).

The inverted CD4:CD8 ratio appears to have clinical significance. In early studies it was shown to be associated with increasing disease stage ((Mills & Cawley, 1982; Herrmann *et al*, 1982)) and development of hypogammaglobulinemia ((Platsoucas *et al*, 1982)). The first indication that it might have prognostic value was from an Italian study of 20 untreated patients with stable indolent disease, where "normal" CD4:CD8 ratios (>1) were one of the factors associated with disease stability (Guarini *et al*, 2003). This has since been supported by British (n=110) and Chinese (n=234) studies of untreated early stage patients that found inverted CD4:CD8 ratios were associated with shorter time to first treatment (TTFT) (Nunes *et al*, 2012) and TTFT and OS (Wu *et al*, 2016) respectively. This was independent of tumour-associated markers such as Zap70 or unmutated IgHV. By contrast a study of 256 Spanish patients demonstrated the opposite; increased CD8 T cell number (i.e. inverted ratio) appeared to associate with improved survival (Gonzalez-Rodriguez *et al*, 2010). However, this study included patients with early and late stage disease and did not directly test CD4:CD8 ratio as a prognostic variable.

Tregs and other CD4 subsets

The increasing availability of phenotypic markers and improvements in flow cytometry has permitted investigation into the activation and differentiation status of T cells, as well as allowing more precise definition of functional subsets (Fig 1). One of the more intensely studied subsets has been regulatory T cells (Tregs), which were originally defined as phenotypically distinct CD4 T cells capable of regulating or suppressing autoimmunity in mice (Sakaguchi *et al*, 2008). These cells became interesting to cancer researchers when they were found to be enriched within solid tumours and to influence anti-tumour responses (Curiel *et al*, 2004).

Several studies have demonstrated increased frequencies of Tregs in CLL patients relative to healthy controls (Motta *et al*, 2005; Piper *et al*, 2011; Biancotto *et al*, 2012; D'Arena *et al*, 2011; Lad *et al*, 2013; Mpakou *et al*, 2017; Rissiek *et al*, 2014; De Matteis *et al*, 2018; Beyer *et al*, 2005; Nunes *et al*, 2012). These studies demonstrated a correlation between increasing frequency and increasing disease stage, however no definitive role for Tregs in disease or response to treatment has been shown to date. The low frequency of Tregs in blood and their phenotypic complexity (Biancotto *et al*, 2012), suggest that differences in laboratory protocols may be important. For example it has been suggested that the increased proportions (%) of Tregs are not a consequence of increased numbers of Tregs but simply reflect the loss of other major CD4 populations (Christopoulos *et al*, 2011).

In common with other cancers, it might be expected that Tregs play their biggest role at sites of disease, however the limited studies on lymph nodes in CLL have provided contradictory findings; no evidence for presence of Tregs (Patten *et al*, 2008) but increased frequency of Tregs in lymph nodes relative to peripheral blood in other studies (Hartmann *et al*, 2015; de Weerd *et al*, 2018). While it is difficult to assign any direct role of Tregs in CLL disease, it is possible that their biggest impact might be indirect. Loss or inhibition of Tregs resulting from drug treatment may contribute to severe adverse autoimmune side-effects (Lampson *et al*, 2016).

There have also been occasional reports of increased systemic frequencies of other CD4 T cell subsets in CLL, including IL-17 secreting T cells (Th17) (Hus *et al*, 2013; De Matteis *et al*, 2018). Increased frequencies of follicular T helper (Tfh) cells, which provide support for B cell development and antibody secretion within germinal centres, have also been reported (Qiu *et al*, 2018; Ahearne *et al*, 2013). Another unusual CD4 T cell subset that occurs at increased frequency in CLL is one that expresses cytotoxic molecules such as granzyme B and perforin (Porakishvili *et al*, 2001). Such cytotoxic CD4 T cells are rare under normal

circumstances but can be grown *in vitro* (Man *et al*, 1990; Appay, 2004) and detected in chronic viral infections and autoimmune diseases (Appay, 2004). Overall, the precise role of these different CD4 T cell subsets in CLL is unclear and remains to be verified with further larger scale studies. However, in non-malignant diseases these CD4 T cell subsets appear to be biomarkers for chronic immune activation, which tempers the view of CLL as an immunosuppressive disease.

Exhausted T cells

T cells that lose their effector functions due to chronic antigen stimulation are defined as “exhausted”. This functional state is most commonly associated with expression of PD-1 (Barber *et al*, 2006; Day *et al*, 2006). T cells expressing PD-1 can be found in solid cancers (Ahmadzadeh *et al*, 2009) and the ligand for PD-1, PD-L1, can be highly expressed in cancer tissue, leading to the idea that blockade of PD-1 could be used as a cancer therapy (Pardoll, 2012). Promising initial results (Brahmer *et al*, 2010) lead to eventual FDA approval of anti-PD1 therapy in melanoma, then subsequently non-small cell lung cancer, head and neck squamous cell carcinoma and classical Hodgkin Lymphoma.

Several studies have shown that an increase in PD-1⁺ T cell frequency in CLL patients (Nunes *et al*, 2012; Riches *et al*, 2013; Palma *et al*, 2017; Brusa *et al*, 2013), although one study demonstrated the reverse (Tonino *et al*, 2012). Despite the increase in PD-1⁺ T cells, there is no global suppression of T cell responses; CMV specific T cells appear to be perfectly functional (Raa *et al*, 2014). The PD-1⁺ T cells in CLL do not appear to have the classical exhaustion phenotype of anti-viral T cells (Riches *et al*, 2013) and have been referred to as being “pseudo-exhausted”. This demonstrates the greater complexity of phenotypes and functions in human T cell populations compared to those found in murine models. Indeed, in healthy individuals PD-1 is expressed on activated T cells which have normal function and do not have an “exhaustion” gene expression signature (Duraiswamy *et al*, 2011). In CLL, there are increased frequencies of activated T cells (Totterman *et al*, 1989) and it is possible that PD-1 expression may be a marker for differentiation state rather than exhaustion (Legat *et al*, 2013).

Studies of exhausted T cells in an animal model of CLL have suggested that anti-PD-1 therapy can restore the function and anti-tumour activity of exhausted T cells (McClanahan *et al*, 2015). However, anti-PD-1 therapy may not succeed in CLL for several reasons. Firstly, it is only effective in certain human cancers. Generally these are characterized by pre-existing immunity to tumour antigens (high frequencies of anti-tumour T cells in the blood or T cell infiltration into the tumour) and high mutation rates to create immunogenic

neoantigens (Seidel *et al*, 2018). As discussed earlier, there is little evidence for strong T cell immunity against CLL. Secondly, truly “exhausted” T cells akin to those found in chronic viral infections have been difficult to find in CLL using only PD-1 as a marker (Riches *et al*, 2013). It is possible that these “exhausted” T cells, if they exist, may express alternative markers or combinations of markers. Thirdly, the rescue of exhausted T cells relies on CD28 expression. As shown by several studies, there is a skewing of CD8 T cell populations in CLL, with high frequencies of differentiated memory cells which often lack CD28, as well as CD27 (Nunes *et al*, 2012; Palma *et al*, 2017; Göthert *et al*, 2013; Tonino *et al*, 2012). Such T cells may be difficult to rescue with antibody therapy even if they express PD-1.

So far there has been very limited testing of anti-PD-1 or PD-L1 therapy in CLL. Interim results presented at conferences have not provided any firm conclusions for efficacy to date (Xu-Monette *et al*, 2018). A trial of anti-PD-1 (Pembrolizumab) therapy in relapsed and Richter transformed (RT) patients demonstrated objective clinical responses in RT patients (4/9) but none in the relapsed CLL patients (n=16) (Table 2, (Ding *et al*, 2017)). It is not clear why there is such a pronounced difference in response in this trial but the authors speculate that genetic events driving the Richter transformation may have created novel antigens recognized by PD-1 expressing T cells.

T cell Differentiation and Ageing

Although it has been difficult to assign a role for any particular T cell subset in CLL, there is no doubt that the disease affects the differentiation of T cells, with a reduction in naive T cells and an increase in highly differentiated memory T cells (Nunes *et al*, 2012; Palma *et al*, 2017; Göthert *et al*, 2013; Tonino *et al*, 2012; Tinhofer *et al*, 2009; Brusa *et al*, 2013; Peller & Kaufman, 1991; Monserrat *et al*, 2013; Serrano *et al*, 1997). This skewing of T cell populations is also a hallmark of ageing (Wikby *et al*, 2008), and while CLL patients are typically elderly, it is clear that the increase in memory T cells in CLL is not just due to age (Nunes *et al*, 2012).

Population studies in Scandinavia show that the inverted CD4:CD8 ratio is present in only 16% of healthy individuals aged 60-94 (Wikby *et al*, 2008), whereas in CLL this can be observed in up to 40% of untreated CLL patients (Nunes *et al*, 2012; Gonzalez-Rodriguez *et al*, 2010). What causes this T cell expansion and differentiation is not known. Some of it can be attributed to CMV infection (Mackus *et al*, 2003; Pourghesari *et al*, 2010) as in the healthy population (Wikby *et al*, 2002), but T cell expansions can also be seen in CMV seronegative CLL patients (Hanna *et al*, 2018; Nunes *et al*, 2012; Pourghesari *et al*, 2010; Riches *et al*, 2013). Furthermore the association between inverted ratio and poorer

prognosis is independent of CMV (Nunes *et al*, 2012; Parry *et al*, 2016). This suggests that CLL cells, or factors associated with CLL, create an inflammatory environment that drives T cell differentiation. This appears to be set early in disease (Nunes *et al*, 2012) and it could be argued that the premature ageing of the immune system in CLL contributes to poorer health (Wikby *et al*, 2008).

The accumulation of memory T cells at the expense of naïve T cells may decrease the capacity of CLL patients to respond to new pathogens, although this is difficult to prove. High frequencies of highly differentiated memory T cells may impact upon treatments that involve activation of the immune system e.g. therapeutic cancer vaccines, checkpoint inhibitors and CAR-T cells. The loss of co-stimulatory molecules such as CD27 and CD28 in highly differentiated memory cells is accompanied by decreased proliferative potential and increased susceptibility to apoptosis. This could compromise adoptive T cell therapies such as CAR-T, which rely on *ex vivo* expansion and then *in vivo* persistence of autologous T cells (Fraietta *et al*, 2018; Rosenberg *et al*, 2011). The development of protocols to optimise CAR-T cell manufacture and efficacy is an area of intense research activity (Orlowski *et al*, 2017), but beyond the scope of this review.

Interpretation of findings in untreated patients

The studies reviewed above on treatment naïve CLL patients illustrate the profound effect of CLL on the T cell compartment and the contrary nature of the disease (summarised in Fig 2). While CLL has historically been defined as an immunosuppressive disease, and the tumour cells themselves are poor antigen presenting cells, there is a paradoxical expansion of the T cell compartment. It is still not clear whether this is driven by CLL antigens, but there are increased frequencies of T cell subsets that normally occur in inflammatory environments. However, it is difficult to associate these discrete functional T cell subsets (Tregs, Th17 and “exhausted” T cells) with poorer prognosis, because the studies are as heterogeneous as the disease itself and produce conflicting results.

The discrepancies between studies may result from a combination of factors including: differences in study cohorts (disease stage, treated or untreated and size of cohort), inclusion of appropriately age-matched controls, lack of consistency in laboratory protocols (particularly for flow cytometry) and the use of freshly isolated versus frozen samples. The majority of the studies have also tested immune parameters as tumour independent markers; small cohort sizes make it difficult to perform subgroup analysis with markers such as Zap70, unmutated Ig VH, p53 mutations or 17p deletions. An important consideration of all studies is that findings with systemic T cells may not be indicative of disease related

events occurring in the lymphoid organs. Nevertheless, the expansion of highly differentiated memory T cells and the development of an inverted CD4:CD8 ratio appear to be two biomarkers in untreated patients (Fig 1) that associate with more aggressive disease.

Impact of treatment on T cells in CLL

The studies reviewed above were population studies with conclusions often based on cross-sectional analysis of patients with different disease classifications or stages. The clearest way to determine the roles of particular T cell subsets is to perform longitudinal studies - these can be linked into clinical trials where serial samples are taken to monitor tumour cells and can also be used to study T cells. One caveat of these studies is that they are seldom performed on untreated or early stage patients, as patients enrolled into trials will have advanced disease and may not be treatment naïve and as such the “baseline” T cell phenotypes will already be skewed. Nevertheless, studies of this sort provide a means to look at the impact of therapy on distinct T cell populations and the kinetics of recovery of T cells to “normal” levels. The studies reviewed here are summarised in Table 2.

Chemotherapy

There is no doubt that recently developed agents that target key pathogenic pathways in CLL will change the way that CLL is treated. However, while these targeted therapies are being implemented, chemotherapy alone, or more usually in combination, remains the standard upfront treatment for CLL worldwide. The cytotoxicity of chemotherapy drugs affects both tumour cells and T cells, with the dramatic loss of T cells an immediate effect of chemotherapy. For example, in patients initially treated with fludarabine both CD4 and CD8 T cells are lost, but there is proportionally a greater loss of CD4 T cells (Fenchel *et al*, 1995; Keating *et al*, 1998). Although there is a recovery in T cell numbers, the counts remain low (below normal levels for CD4) even 2 years after treatment (Keating *et al*, 1998). Despite this persistent T cell suppression, the overall risk of major infections in fludarabine-treated patients was low (<3%) and decreased with time after treatment (Keating *et al*, 1998). More detailed studies of T cell subsets in fludarabine-treated patients demonstrated a reduction in Treg frequency that was not seen with non-fludarabine treatment (Beyer *et al*, 2005). Laboratory studies using human and mouse T cells suggest that the T cells that survive fludarabine treatment are predominantly mature T cells with a Th1 phenotype (Gassner *et al*, 2011). This supports the concept that memory T cells are preferentially retained after therapy, however more detailed phenotyping to assess differentiation status was not done.

Fludarabine-cyclophosphamide-rituximab (FCR) combination is a commonly used chemoimmunotherapy regimen that has higher clinical response rates than fludarabine alone, but an increased rate of major infections (~10%) early in treatment (Tam *et al*, 2008). Not surprisingly this combination was found to result in T cell loss (both $\alpha\beta$ and $\gamma\delta$ T cells) with a sustained drop in CD4 T cell counts in particular (Ysebaert *et al*, 2010). Lower median CD4 counts were associated with stable Minimal Residual Disease (MRD) levels of <1% over 24 months (defined by flow cytometric detection of CLL cells) (Ysebaert *et al*, 2010). By contrast higher median CD4 counts were associated with progressive MRD of >1%, suggesting a negative effect of CD4 T cell recovery on disease control (Ysebaert *et al*, 2010).

Bendamustine is used as a chemotherapy alternative for CLL patients unsuitable for fludarabine combinations and is often used in combination with rituximab. Patients treated with this regimen for more than 36 months demonstrated similar effects on T cell counts to fludarabine-based regimens, with CD4 T cells being disproportionately affected and slower to recover back to normal numbers (Martínez-Calle *et al*, 2018). In this study, there was a high rate of major infections requiring hospitalisation (49%), and this was associated with the delayed recovery of CD4 T cell counts (Martínez-Calle *et al*, 2018). Neither fludarabine or bendamustine appear to affect NK cell numbers (Ysebaert *et al*, 2010; Martínez-Calle *et al*, 2018).

Overall, studies on chemotherapy have shown profound T cell depletion and slow T cell recovery. However, the deeper phenotypic profiling of T cell populations, as done in untreated patients (Table 1), has not been carried out. This may in part be due to pragmatic reasons, as chemotherapy greatly reduces the number of T cells available to study. What is clear is that the disproportionate effect of chemotherapy on reduction and recovery of CD4 T cell numbers means that studies on the proportions of CD4 subsets or CD4:CD8 ratio in treated patients need careful interpretation.

Non-chemotherapy treatments

Lenalidomide

Lenalidomide belongs to a class of immunomodulatory drugs related to thalidomide. It has been tested in CLL patients (Chanan-Khan *et al*, 2006) but is not currently approved for therapy. This is largely because of the severe side-effects associated with drug treatment, particularly haematological side effects and tumour lysis syndrome, although this appears to depend on dose and the type of patient treated (Chanan-Khan *et al*, 2006; Ferrajoli *et al*,

2008). Conceptually lenalidomide is an attractive drug for CLL, as laboratory studies have demonstrated that it can augment T cell immunity, overcoming CLL defects in immune synapse formation with T cells (Ramsay *et al*, 2008). Given this mode of action, it is not surprising that there have been several studies on the impact of lenalidomide treatment on T cells (summarised in Table 2). Surprisingly, lenalidomide can reduce T cell (as well as B and NK cell) numbers immediately after initiating treatment but these recover to pre-treatment levels by the end of 3 weeks (Aue *et al*, 2009). It also has an overall activating effect on T cells (Aue *et al*, 2009; Winqvist *et al*, 2017), resulting in increased frequencies of proliferating and effector memory T cells with an inflammatory cytokine profile (Winqvist *et al*, 2017; Aue *et al*, 2018). More dramatic effects might be predicted in the lymphoid organs, however the limited studies on T cell populations in lymph nodes of CLL patients have not demonstrated significant changes post treatment (Aue *et al*, 2018; 2009). Overall, these studies have not revealed a clear link between T cell activation and response to treatment.

Effects on T cell populations have also been seen when lenalidomide has been used at lower doses as an adjunct therapy to ofatumumab (Vitale *et al*, 2016) or rituximab (Egle *et al*, 2018). Low doses of lenalidomide given after ofatumumab treatment of relapsed/refractory patients resulted in durable clinical responses and a decrease in overall T cell counts. High baseline CD4 T cell and NK cell counts were associated with complete response (Vitale *et al*, 2016). By contrast, a trial using escalating doses of lenalidomide added to fludarabine/rituximab treatment showed that patients who could tolerate >5mg/day lenalidomide had lower frequencies of “exhausted” PD-1⁺ CD4 memory T cells, although no association was found with response to treatment or PFS. In these contrasting studies, it was difficult to dissect out the impact of low dose lenalidomide on T cells and further laboratory investigations are needed.

Venetoclax

Venetoclax is small molecule inhibitor of BCL-2, which has demonstrated clinical efficacy in relapsed or refractory CLL (Roberts *et al*, 2016) and has the potential to be used as a frontline treatment. It has also been proposed as a potential salvage therapy for patients who relapse or discontinue treatment with idelalisib or ibrutinib (Eyre *et al*, 2019)(see below). Laboratory investigation of venetoclax demonstrated that it does not affect activated memory T cells, nor T cell function overall, but does reduce the absolute numbers of naïve T cells (Mathew *et al*, 2018). *Ex vivo* analysis of samples from patients treated with venetoclax demonstrated a maintenance of absolute T cell numbers, but a decrease in proportion and absolute number of Treg cells. There was also an effect on the CD8 compartment, with fewer CD8⁺PD-1⁺ T cells and lower proportions of CD8 T cells capable of secreting IFN γ and

TNF α (de Weerd *et al*, 2018). Although further studies are needed to validate these results, they suggest the targeting of CLL cells by venetoclax has a beneficial effect on the T cell compartment.

Idelalisib

Idelalisib is a small molecule inhibitor of PI3K- δ that was developed to disrupt the pro-survival pathways of B cells and, in combination with anti-CD20 monoclonal antibodies, has shown efficacy in relapsed refractory disease in CLL (Furman *et al*, 2014). However upfront use of idelalisib has resulted in a high incidence of adverse side effects, including enterocolitis and transaminitis (Furman *et al*, 2014). Although idelalisib is not directly cytotoxic to T cells, it might have other effects that could disrupt T cell regulation and promote activation of autoimmune T cells (Ali *et al*, 2014). In support of this hypothesis, *in vitro* testing of idelalisib with healthy donor (Herman *et al*, 2010) and CLL (Martinelli *et al*, 2018) derived T cells has demonstrated an inhibitory effect on T cell secretion of inflammatory cytokines such as TNF α , IL6, IL10 (Herman *et al*, 2010) and IFN γ (Martinelli *et al*, 2018). Furthermore decreased numbers of Treg cells were found in patients with idelalisib-induced hepatotoxicity (Lampson *et al*, 2016). Interestingly these patients were younger and had mutated IgHV, which would usually be considered as favourable prognostic markers for CLL. In a follow up study, higher resolution flow cytometry was used to define two CD4 populations that changed in frequency after treatment with idelalisib (Vartanov *et al*, 2018). One of these was a population of CD4⁺CD39⁺ Treg cells, which decreased in frequency in patients with hepatotoxicity (Vartanov *et al*, 2018). Larger numbers of patients need to be studied to validate these findings, however *ex vivo* monitoring of T cell subset frequency and function might be useful in elucidating mechanisms contributing to autoimmunity and susceptibility to infection in CLL.

Ibrutinib

Ibrutinib is a small molecule inhibitor of Bruton's tyrosine kinase (BTK), that has demonstrated marked efficacy and was initially well tolerated in clinical testing (Brown, 2018). Although ibrutinib is targeted towards signalling pathways in B cells, it may also have systemic effects on T cells through direct or indirect mechanisms. One of the key effects of treatment is transient lymphocytosis caused by efflux of CLL cells from the lymphoid tissues such as bone marrow, lymph nodes and spleen. This efflux of CLL cells is accompanied by a decrease in absolute numbers of peripheral T cells from pre-treatment levels (Burger *et al*, 2018), including both CD4 and CD8 subsets (Yin *et al*, 2017; Niemann *et al*, 2016). This decrease returns absolute counts to the normal range, however it appears that the timing of

samples was important. The first sample time point for these studies was 12-24 weeks post treatment (Yin *et al*, 2017; Niemann *et al*, 2016; Burger *et al*, 2018), whereas a study using samples from 8 weeks post-treatment demonstrated a transient increase in T cell counts followed by a decrease at the next time point of 20 weeks post-treatment (Long *et al*, 2017). This expansion was predominantly in the more differentiated memory T cells (EM and EMRA)(Long *et al*, 2017).

Sequencing of T cell receptor beta chain genes (TCR β) of systemic T cells collected pre- and post-therapy demonstrated that ibrutinib treatment could change the TCR β repertoire. By implication, ibrutinib had selected for the expansion of certain T cell clones that were present at low frequency prior to treatment (Yin *et al*, 2017).

Besides the changes in numbers of T cells, ibrutinib also appears to have effects on T cell phenotype, with reduced expression of activation markers (Niemann *et al*, 2016) and check-point inhibitors such as PD-1 and CTLA-4 (Niemann *et al*, 2016; Long *et al*, 2017). Further studies are needed to determine whether any of the changes in T cell populations post ibrutinib treatment can act as biomarkers to predict the longevity of clinical responses to ibrutinib or adverse side effects. Nevertheless, these changes have led to suggestions that, in addition to its direct action against CLL cells, ibrutinib could also be used to boost immunotherapeutic approaches such as CAR-T therapy (Fraietta *et al*, 2016).

Conclusions and Future Prospects

This review has highlighted how CLL disease and treatment can affect the composition and function of T cell populations. The plethora of T cell abnormalities observed in CLL appear to be unique among human cancers. More than 40 years after the first description of T cell abnormalities in CLL, it is still not clear whether T cells are observers or participants in disease development. This requires knowledge of function in the context of disease, however direct measurement of T cell function is hampered by the lack of robust tumour antigens, and often relies on rather contrived *in vitro* assays. Paradoxically it may be easier to show that T cells contribute to adverse autoimmune events in CLL.

The advent of more powerful technology such as mass cytometry (CYTOF) and single cell RNA sequencing, and an increasing appreciation of T cell metabolism, will allow study of T cells with greater precision. But will this lead to more observational studies that fail to provide mechanistic insights? In the future, it is likely that the real breakthroughs in understanding the role of T cells in CLL will come from comparative studies of T cells in the blood and lymphoid organs and assessing T cell parameters in long-term longitudinal

studies. In the age of big data, it is likely that these future studies will combine immunological data with many other clinical and non-clinical parameters in a systems approach to CLL research. These studies are also likely to reveal whether non-conventional T cells or cells of the innate immune system play a role in disease development or response to therapy.

In the meantime, how can information about T cells be useful in CLL? Knowledge about the differentiation status, function and frequency of particular T cell subsets in the blood can provide a snapshot of immune health. This approach might be useful in at least 3 different scenarios:

1. **In early stage patients who are not receiving treatment but are at risk of disease progression.** The inverted CD4:CD8 ratio and the accompanying expansion of highly differentiated memory cells can define patients with more aggressive disease (Table 1). Therefore, a T cell signature based on threshold frequencies of certain T cell subsets may help to predict which patients will develop progressive disease. While early treatment of CLL with chemotherapy has not shown any clear benefit, the advent of more targeted, lower toxicity therapies could make earlier treatment more feasible.
2. **In patients undergoing treatment.** Changes in the frequencies of certain T cell subsets before and during treatment may allow prediction of which patients will respond or develop adverse side effects/infections (Table 2). The increasing use of immunotherapies in CLL suggest that monitoring of T cell populations will continue. For CAR-T cells, this may also determine which patients get selected for expansion therapy, excluding those with an unfavourable T cell profile (high frequencies of late memory T cells). The detrimental changes in T cell profiles as disease progresses (Table 1) also suggests the possibility that patients diagnosed with early stage disease and a good immune profile, could store samples of their blood as an insurance policy for future use.
3. **In patients post-treatment.** The efficacy of chemo-immunotherapy regimens and the less toxic new targeted therapies suggests the prospect of long-term control of CLL. The consequence of this is an increasing population of elderly CLL patients with stable disease, but with sub-optimal immune systems due to incomplete recovery of T cell populations (Table 2) or other changes in T cell subsets. These patients are at increased risk of infection and monitoring of T cell populations may identify the patients that are most susceptible. More precise measurement of pathogen-specific T cells e.g. influenza-specific, may also allow grouping of the patients most likely to respond to vaccinations (Wagar *et al*, 2011).

T cells remain one of the most intensely studied cells in the human body because of their important role in many human diseases (Su *et al*, 2013). For CLL, research on the behaviour and function of T cells will continue to be important, both for improving T cell-based therapies and as a means of monitoring immune health in patients before, during and after treatment.

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Figure Legends

Figure 1. The phenotypic and functional complexity of Human T cells.

The phenotypic and functional complexity of human T cells. Conventional human T cells can be classified according to their HLA restriction (CD4 or CD8), their differentiation status, function and original location (blood or lymphoid tissue) (Farber *et al*, 2014). After antigen priming e.g. during viral infection, naive T cells become activated and acquire effector function (cytokine secretion and/or cytotoxicity) The bulk of effectors die off, leaving behind a smaller pool of T cells that differentiate into distinct memory T cell subsets. Persistent chronic antigen stimulation leads to accumulation of terminally differentiated effector memory T cells (EMRA), which have a limited potential for proliferation. T cells can also become “exhausted” and lose their function or become senescent and lose their capacity to proliferate.

Figure 2. T cell abnormalities in CLL.

A. Schematic summary of relative differences in the proportion of T cell subsets between healthy controls and CLL patients in blood. T cells in CLL have an inverted CD4:CD8 ratio and a skew towards highly differentiated memory T cells. Note that CLL cells typically outnumber T cells at far higher ratios than shown. B. Phenotypical and functional features of T cells in CLL. These generally have a more activated phenotype (CD69,HLA-DR expression) with frequent expression of checkpoint inhibitor molecules (PD-1, CTLA-4) and markers of differentiation (CD57). CLL cells express PD-L1 and CD86 and have disordered F actin microfilament structures that contribute to defects in immune synapse formation.